Toll-like Receptor 4 Polymorphisms (rs4986790, rs4986791) and Serum Tumor Necrosis Factor Alpha levels in Chronic Obstructive Pulmonary Disease and Lung Cancer

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Abstract

Background: Genetic factors and environmental conditions have a great impact on Chronic Obstructive Pulmonary Disease (COPD) and lung cancer (LC). Toll Like Receptor 4 (TLR4) gene polymorphisms may induce airway inflammation and cancer. Elevated TNF-α serum levels in COPD and LC patients exacerbate inflammation and disease progression.

Objectives: To investigate the prevalence and type of TLR4 polymorphisms and TNF-α serum levels in COPD and LC susceptibility.

Patients and methods: Case-control study with 50 COPD, 50 LC, and 50 healthy controls aged 19–75. DNA was extracted from blood samples, and PCR-RFLP and ELISA were used to evaluate TLR4 polymorphisms (rs4986790, rs4986791) and serum TNF-alpha.

Results: Most LC patients (86%) were male compared to COPD (58%), P = 0.008. Smokers were 68% and 22% in LC and COPD patients, respectively, compared to 32% in controls (P < 0.001). Significantly, 38% of COPD patients were exposed to avian excreta compared to 0% of controls (P < 0.001). Genotyping of 896 A/G (SNP rs4986790) showed that 2% of LC patients were heterozygote with insignificance (P = 0.315) compared to control and COPD, and 4% were heterozygotes (CT) for 1196 C/T (SNP rs4986791) with insignificant (P = 0.153).

Conclusion: The genotype (AA) of TLR4 (896 A/G) represents 98% and the genotype (CC) of TLR4 (1196 C/T) represented 96% of lung cancer cases. In COPD, both the genotype (AA) of TLR4 (896 A/G) and (CC) of TLR4 (1196 C/T) represented 100%. TNF-α had a significant negative correlation with chemotherapy.

Keywords: Toll-like receptor 4; TNF-α; COPD; Lung cancer.

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Introduction

Chronic obstructive pulmonary disease (COPD) and lung cancer (LC) are major global health issues, significantly impacting morbidity and mortality. These diseases arise from complex genetic and environmental interactions, with smoking being a critical risk factor. However, not all smokers develop COPD or LC, indicating a genetic predisposition's crucial role. Research suggests that smokers with COPD have a higher risk of developing LC, implying a potential genetic link **(Szalontai et al., 2021; Uliński et al., 2022).**

Airway inflammation is key in COPD and LC pathogenesis **(Tan et al., 2021; Kotlyarov, 2022)**. Toll-like receptors (TLRs), especially TLR4, are vital to the innate immune system. They detect pathogen-associated molecular patterns, triggering immune responses and cytokine production. Polymorphisms in the TLR4 gene, such as rs4986790 and rs4986791, may alter immune responses, contributing to chronic inflammation and carcinogenesis. Understanding these polymorphisms' roles in COPD and LC is crucial for identifying high-risk individuals and developing targeted therapies **(Wicherska-Pawłowska et al., 2021).**

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine integral to the inflammatory response. Produced primarily by macrophages, TNF-α levels are elevated in the bronchoalveolar lavage fluid of COPD and LC patients. Its role in maintaining airway inflammation is critical to both diseases' pathogenesis. **(Gong et al., 2021; Laha et al., 2021).**

The aim of this study was to explore the prevalence of TLR4 polymorphisms (rs4986790 and rs4986791) and the TNF-α serum levels in COPD and LC.

Patients and methods

This case-control study was conducted from August 2022 to September 2023 at the Chest and Oncology Departments of Qena University Hospitals, South Valley University. The study included 50 patients with COPD, 50 patients with LC from which 45 had chemotherapy, and 50 healthy controls of matching sex and age, all living in the same geographic area. Participants were aged 19 to 75 years. COPD and LC diagnoses followed the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines **(Gómez and Rodrigue, 2002).**

Sample size justification: The sample size was calculated based on **Kurt et al., 2016** study where the prevalence of CC genotype of TLR4 1196 C/T was 97.5% in the LC group and 91% in the controls. Epi Info was used to calculate the sample size by considering the following assumptions: - 95% two-sided confidence level, with a power of 80%. & An error of 5% odds ratio calculated= 1.115. The final maximum sample size taken from the Epi- Info output was 50 for each group.

Written informed consent was obtained from each patient before enrollment in the study. All patients underwent history taking including age, sex, marital status, occupation, special habits, medical history for associated comorbidities. Clinical examination focused on local chest examination, routine laboratory investigations included complete blood count, liver and renal function tests, and chest X-rays. Patients with other chest diseases such as bronchial asthma, bronchiectasis, or those who did not consent were excluded.

For TLR4 rs4986790 and TLR4 rs4986791 genotyping, three milliliters of peripheral venous blood were collected and placed into sterile tubes containing ethylenediaminetetraacetic acid (EDTA) for DNA extraction by the salting out method as described previously by **(Miller et al., 1988).** Two milliliters of peripheral venous

blood were collected into sterile plain tubes to obtain serum for analysis of TNF-alpha concentration. Both extracted DNA and serum samples were preserved at -20 ˚C until molecular and ELISA analysis.

Genotyping of TLR4

The TLR4 (SNP rs4986790) gene was amplified using forward primer 5′-AGCATACTTAGACTACTACCTCCATG-3′ and reverse 5′-GAGAGATTTGAGTTTCAATGTGGG-3′ (Invitrogen, USA). The TLR4 (SNP rs4986791) gene was amplified using primers 5′-GGTTGCTGTTCTCAAAGTGATTTTGG GAGAA-3′ (forward) and 5′-GGAAATCCAGATGTTCTAGTTGTTCT AAGCC-3′ (reverse) (Invitrogen, USA).

DNA extraction: PCR volume was 25 µl for both genes as follow: 12.5 µl master mix (New England BioLabs, USA), 1 µl forward primer, 1 µl reverse primer, 2 µl of extracted DNA, and 8.5 µl H2O. The amplification conditions for both genes were; initial denaturation at 94 °C for 30 seconds, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58.5 °C for 1 minute, and extension at 72 °C for 30 seconds, with a final extension at 72 °C for 7 minutes as described by **(Iqbal et al., 2017).**

PCR products were separated by gel electrophoresis using 2% agarose gel containing 5% ethidium bromide twice: once to confirm the presence of PCR product and again to determine the genotypes after restriction fragment length polymorphism (RFLP).

For detection of the TLR4 genotype (SNP rs4986790) 2 µl of restriction enzyme Nco I (Thermo Fisher Scientific Inc., Waltham, MA) was added to 8 µl of PCR product, 2.5 µl of NE Buffer, and 12.5 µl of H2O, Then incubation at 37 °C for 1 hour. Genotyping was classified as follows AA genotype (188 base pairs (bp)), AG genotype (188 bp, 168 bp, and 20 bp), and GG genotype (168 bp, and 20 bp).

For detection of the TLR4 genotype (SNP rs4986791), 2 µl of restriction enzyme Hinf I (New England BioLabs) in a condition similar to SNP rs4986790. Genotyping was classified as follows CC genotype (124 base pairs (bp)), CT genotype (124 bp, 98 bp, and 26 bp), and TT genotype (98 bp, and 26 bp).

PCR products were separated by horizontal gel electrophoresis (Cleaver Scientific, UK) using 2% agarose gel containing 5% ethidium bromide. Bands are visualized by the UV Gel Documentation System (MicroDoc System, UK).

Determination of serum TNF Alpha Concentration

Estimation of serum TNF alpha levels using a solid phase enzyme-linked immunosorbent assay kit (Biospes TNF-alpha ELISA Kit - (Catalog No. ELISA-TNF-001, Biospes, Chongqing, China) according to the manufacturer's instructions. The kit had a detection range of 7.81-500 pg/mL, and both intra-CV and inter-CV are < 10%.

The study was approved by the Ethics Committee, Qena Faculty of Medicine, South Valley University and conducted in accordance with the Declaration of Helsinki.

Ethical approval code: SVU-MED-MIC007-4-24-7-892.

Statistical analysis

Data were collected, coded, revised, and entered into IBM SPSS version 27. Categorical variables were presented as numbers and percentages, while numerical variables were shown as means and standard deviations. Data normality was assessed using the Shapiro-Wilk test. The chi-square test was used to compare qualitative variables between cases and controls. The independent t-test compared parametric numerical variables, while the Kruskal-

Wallis test was applied for non-parametric data across two or more groups and fisher exact test for comparing between two groups when parameter prevalence among one group is zero. one way analysis of variance (ANOVA) test was used when comparing multiple groups. A 5% margin of error and a 95% confidence interval were used, with $p \leq 0.05$ considered significant.

Results

All groups showed male predominance, with most participants married. In the LC group, employees were most common (46%), while housewives were most frequent in the COPD (44%) and control (26%) groups. Smoking was highest in the LC group (68%), with ex-smokers prevalent in the COPD group (32%), and non-smokers in the control group (64%). Age in the control group was significantly lower (**Table.1).**

Table 1.Sociodemographic features of the studied groups

Parameters (N (%))		Lung cancer (50)	COPD (50)	Controls (50)	P-value
		Number (%)	Number (%)	Number (%)	
Gender	**Male**	43 (86%)	29 (58%)	35 (70%)	0.008*
	Female	7 (14%)	21 (42%)	15 (30%)	
Marital status	**Single**	2 (4%)	1 (2%)	8 (16%)	0.015*
	Married	48 (96%)	49 (98%)	42 (84%)	
Occupation	**Housewife**	7 (14%)	22 (44%)	13 (26%)	<0.001*
	Farmer	12 (24%)	9 (18%)	7 (14%)	
	Employee	23 (46%)	5 (10%)	12 (24%)	
	Worker	3 (6%)	9 (18%)	7 (14%)	
	Student	0 (0%)	0 (0%)	3 (6%)	
	Querry worker	1 (2%)	2 (4%)	2 (4%)	
	Aluminum factory	2 (4%)	0 (0%)	0 (0%)	
	Lab technician	1 (2%)	0 (0%)	3 (6%)	
	Engineer	0 (0%)	0 (0%)	2 (4%)	
	Physician	0 (0%)	0 (0%)	1 (1%)	
	Health supervisor	0 (0%)	1 (2%)	0 (0%)	
	Carpenter	0 (0%)	1 (2%)	0 (0%)	
	Cleaning worker	1 (2%)	0 (0%)	0 (0%)	
Smoking status	**Non-smoker**	4 (8%)	13 (26%)	32 (64%)	< 0.001*
	Current smoker	34 (68%)	11 (22%)	16 (32%)	
	Ex-smoker	9 (18%)	16 (32%)	0 (0%)	
	Passive smoker	3 (6%)	10 (20%)	2 (4%)	
		Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Age (years)		65.120 ± 11.3	61.240 ± 6.66	35.840 ± 9.270	<0.001[F]

*Chi-square test, F: ANOVA test.

Hypertension was the most prevalent comorbidity in the LC (24%) and COPD (22%) groups, while diabetes mellitus was most common in controls (12%). Oxygen saturation was higher in the controls and lowest in the COPD group. Cough predominated in LC and COPD cases, followed by dyspnea and chest tightness.

Among controls, chest tightness was more frequent than dyspnea, **(Table.2).**

Table 2. Clinical data in the studied groups

Parameters (N (%))		Lung cancer (50)	COPD (50)	Controls (50)	P value
		Number (%)	Number (%)	Number (%)	
Associated morbidities	**Diabetes mellitus**	7 (14%)	3 (6%)	6 (12%)	<0.001*
	Hypertension	12 (24%)	11 (22%)	2 (4%)	
	Diabetes and HTN	9 (18%)	6 (12%)	1 (2%)	
	CKD and HTN	1 (1%)	0 (0%)	1 (1%)	
	Cardiac disease	1 (1%)	2 (4%)	0 (0%)	
	Bronchial asthma	0 (0%)	0 (0%)	1 (2%)	
	Chronic kidney disease	0 (0%)	3 (6%)	0 (0%)	
	Breast cancer	1 (2%)	0 (0%)	0 (0%)	
Clinical data	**Cough**	31 (62%)	33 (66%)	9 (18%)	0.132
	Hemoptysis	19 (38%)	9 (18%)	2 (4%)	
	Chest tightness	25 (50%)	21 (42%)	6 (12%)	
	Dyspnea	29 (58%)	23 (46%)	4 (8%)	
	Recurrent chest infection	17 (34%)	4 (8%)	3 (6%)	
	Wheeze	22 (44%)	14 (28%)	3 (6%)	
	Fatigue	33 (66%)	16 (32%)	4 (8%)	
	Loss of weight	25 (50%)	4 (8%)	3 (6%)	
	Hoarseness	14 (28%)	4 (8%)	2 (4%)	
		Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Oxygen saturation (%)		90.0 ± 2.51	86.90 ± 8.11	95.12 ± 0.32	< 0.001[F]

*Chi-square test, **Fisher's exact test, F: ANOVA test.

Non-small lung cancer type was in 35 (70%) from which 18 (36%) were adenocarcinoma, 12 (24%) were squamous cell carcinoma and 5 (10%) were large cell carcinoma. 19 (38%) had stage II LC, 14 (28%) had stage III and 17 (34%) had stage I. 45 (90%) underwent chemotherapy. 17 (34%) had well differentiated tumor with grade I and 13 (26%) had moderately differentiated tumor with grade II and the rest had poor (11 (22%)) and undifferentiated tumors (9 (18%)), **(Table 3).**

Table 3. Tumor characters among lung cancer patients

Variables	Lung cancer (50)
Lung cancer types	
• **Small cell lung cancer**	15 (30%)
• **Non-small cell lung cancer:**	35 (70%)
- **Adenocarcinoma**	18 (36%)
- **Squamous Cell Carcinoma**	12 (24%)
- **Large Cell Carcinoma**	5 (10%)
Tumor grade	
• **I**	17 (34%)
• **II**	13 (26%)

• **III**	11 (22%)
• **IV**	9 (18%)
Stage	
• **I**	17 (34%)
• **II**	19 (38%)
• **III**	14 (28%)
Treatment received	
• **Chemotherapy**	45 (90%)
• **No treatment received**	5 (10%)

AA genotype, A-allele, CC genotype and C-allele were the predominant among the LC and control groups (**Table.4).** All COPD patients and controls had the AA genotype for SNP rs4986790 and the CC genotype for SNP rs4986791. (**Table .5)** and **Fig.1** showed electrophoretic patterns.

Table 4. TLR 4 (SNP rs4986790 and SNP rs4986791) genotyping in lung cancer patients and control group

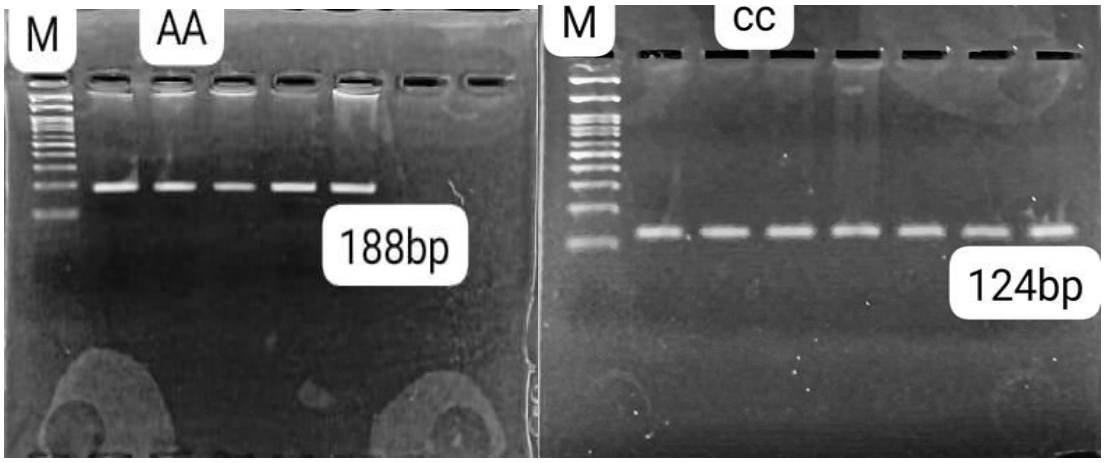
Genotype (N (%))		Lung cancer (50)	Controls (50)	P value	Odds ratio (95% CI)
Genotypes 896 A/G (SNP rs4986790)	**AA**	49 (98%)	50 (100%)	0.315[f]	1 (reference)
	AG	1 (2%)	0 (0%)		0.980 (0.942-1.020)
Alleles (SNP rs4986790)	**A-allele**	99 (99%)	100 (100%)	0.316[f]	1 (reference)
	G-allele	1 (1%)	0 (0%)		0.990 (0.971-0.1010)
Genotypes 1196 C/T (SNP rs4986791)	**CC**	48 (96%)	50 (100%)	0.153[f]	1 (reference)
	CT	2 (4%)	0 (0%)		0.960 (0.907-1.016)
Alleles (SNP rs4986791)	**C-allele**	98 (98%)	100 (100%)	0.155[f]	1 (reference)
	T-allele	2 (2%)	0 (0%)		0.980 (0.953-1.008)

f: fisher exact test.

Table 5. Distribution of Genotypes of COPD and Control Group

Genotype (N (%))		COPD (50)	Controls (50)	P value
TLR4 896 A/G genotypes (SNP rs4986790)	**AA**	50 (100%)	50 (100%)	1.00 [f]
	AG	0 (0%)	0 (0%)	
TLR4 1196 C/T genotypes (SNP rs4986791)	**CC**	50 (100%)	50 (100%)	1.00 [f]

f: fisher exact test.



(A) **(B)**

Fig.1. (**A**) the electrophoretic patterns corresponding to genotype TLR4 896 A/G (SNP rs4986790) among COPD and LC cases (AA genotype 188 bp). (**B**) The electrophoretic patterns corresponding to genotype TLR4 1196 C/T genotypes (SNP rs4986791) among COPD and LC cases (CC genotype 124 bp)

TNF- α was highest among LC patients without chemotherapy followed by COPD patients and was lowest among LC patients who had chemotherapy (P < 0.001) **(Table.6).**

Table 6. TNF-α in the studied groups

Variables	Lung cancer without chemotherapy	Lung cancer with chemotherapy	COPD	Controls	P-value
Tumor necrosis factor α	23.14 ± 2.68	5.12 ± 2.37	8.08 ± 4.1	7.57 ± 5.15	<0.001*

*Kruskal-Walli's test

Significant association of TNF-α levels on the clinical response to chemotherapy in LC patients (r=−0.520 and P<0.001). **(Fig.2).**

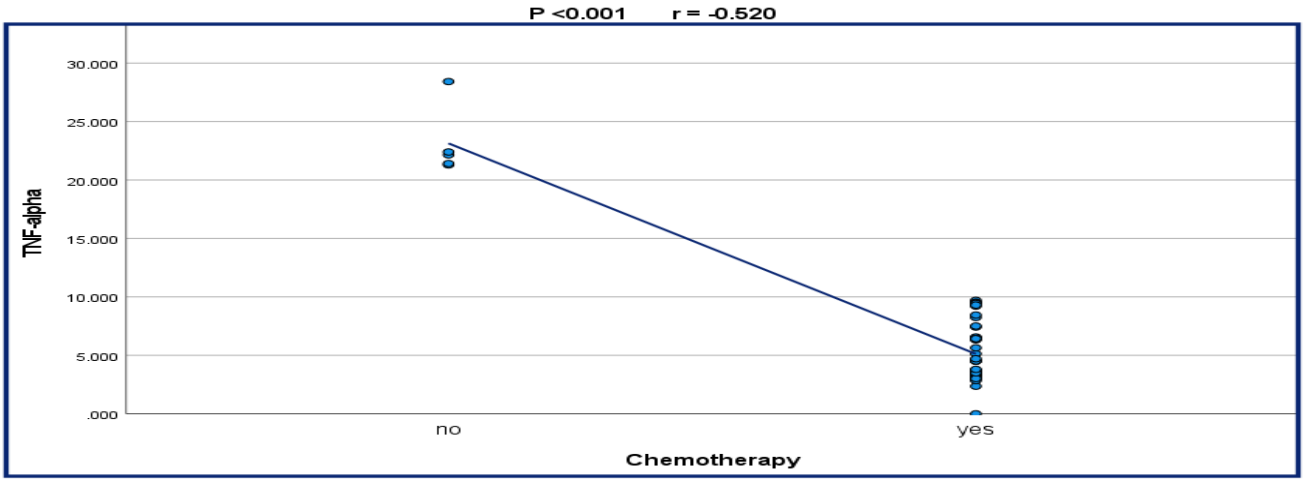


Fig. 2. TNF-α levels in clinical response to chemotherapy in LC patients

Discussion

In our result, most of LC patients were males (86%), compared to (58%) in COPD, and smokers were 68% in LC patients and 22% in COPD. LC and COPD were significantly detected in older persons (65.12 ± 11.3 in LC and 61.24 ± 6.66 in COPD). Males smoke more and work in respiratory-hazardous industries, including construction, mining, and manufacturing, which contribute to these diseases. Older people are more likely to develop COPD and LC due to years of smoking, pollution, and job dangers. Our results agreed with **Budulac et al. (2012)** concluded that most COPD patients were male (86.8%) with an average age of 61.6 years (± 7.7) and 63.2% were smokers. Also, **Yu et al. (2016)** found that 78.6% of COPD patients were male, with an average age of 65.73 years (± 9.75). 71.2% were smokers.

In our study, most LC patients were farmers or industrial workers. They had higher age (65.12±11.30) than controls (35.84±9.27). These findings support by **Bonner et al. (2017),** who found a strong connection between LC incidence and age in farmers and commercial pesticide applicator groups. **Hosseini et al. (2022)** found higher odds ratios (ORs) for lung cancer in male construction workers, petroleum industry workers, female farmers, and female bakers. LC risk factors include age, genetic predisposition, and occupational exposure.

Lung cancer patients were more likely to be smokers (68% vs. 32%) and had hypertension and diabetes. Their reduced oxygen saturation indicated significant respiratory damage. A meta-analysis by **Almatrafi et al. (2023)** estimated patient comorbidity prevalence, corroborating our findings. Hypertension was 35.2%, COPD 23.5%, ischemic heart disease (IHD) 16.6%, and diabetes 12.5%. These comorbidities complicate LC treatment and patient outcomes.

Although our analysis did not identify significant relationships between TLR4 SNPs and LC risk, **Kurt et al. (2016)** and **Ding et al. (2017)** highlight the complexity of genetic variables in LC susceptibility. Similar to our result, TLR4 rs4986790 (Asp299Gly) genotype frequencies were similar between controls and LC patients. **Ding et al. (2017)** found that rs4986791 (Thr399Ile) was significantly different; the CT genotype of rs4986791 had a 3.857-fold higher risk of lung cancer than the CC genotype. Allele frequency is different between different populations. Our study and the previous studies analyzed different populations. **Ding et al. (2017)** found the two TLR4 SNPs reduced cancer risk. The odds ratio (OR) of the allele model for rs4986791 was 0.764, while the odds ratio (OR) of the allele model for TLR4 rs4986790 was 0.871.

TLR4 genotype frequencies did not change between COPD patients and controls, suggesting they may not affect COPD susceptibility or development.

Toll-like receptors (TLRs), notably TLR4, have been widely studied in cigarette smoke-induced inflammation. **Karimi et al. (2006), Pace et al. (2008), Sarir et al. (2009),** and showed TLR4's participation in cigarette smoke-induced cytokine production and lung inflammation. **Maes et al. (2006)** found that TLR4 deletion in mice decreased cigarette smoke-induced inflammation indicators, underscoring its importance in lung inflammation.

In contrast to our findings, **Kanwar et al. (2021)** found significant relationships between heavy smokers' TLR4 polymorphisms (TLRD299G and TLR4T3991) and COPD development. The TLR4T3991 polymorphism enhanced COPD risk and infection susceptibility, whereas the D299G variation lowered COPD severity due to its lipopolysaccharide hypo-reactivity. This shows the complicated

significance of TLR4 genetic polymorphisms in COPD outcomes, which vary by environment and genetics. **McKelvey et al. (2016)** noted that TLRs have a dual function in pulmonary inflammation, complicating COPD treatment methods targeting these receptors.

Numerous meta-analyses have explored the link between TNF-α levels and COPD, with inconsistent results. In a meta-analysis of 24 studies, **Su et al. (2016)** showed no connection between TNF-α levels and COPD. **Gan et al. (2004)** found a link between systemic inflammatory markers, such as TNF-α, and reduced lung function in COPD in a meta-analysis of 14 studies. Further research is needed to establish the function of TNF-α in COPD pathogenesis across different groups due to the inconsistent study outcomes.

As our result, **Yao et al. (2019)** found higher TNF-α levels in COPD patients compared to healthy controls. They suggested that COPD patients' genetic or constitutional variations from controls predispose them to systemic and pulmonary inflammation. In COPD, inflammatory mediators such as IL-8, IL-6, and TNF-α exacerbate lung inflammation and damage. Smoking, oxidative stress, and tissue hypoxia may cause local pulmonary inflammation and raise blood inflammatory markers, leading to COPD pathogenesis **(Bezerra et al., 2023).**

According to **Gao et al. (2015),** TNF-α plays a crucial role in airway remodeling in COPD. TNF-α increases matrix metalloproteinase synthesis by airway cells and fibroblasts, causing extracellular matrix breakdown and lung parenchyma damage in emphysema. TNF-α also enhances tissue fibrosis and lung remodeling by promoting fibroblast differentiation into myofibroblasts.

In our result, TNF α was significantly higher in LC cases without chemotherapy versus those received chemotherapy. **Ferrajoli et al., (2002)** concluded that TNF α level decrease during chemotherapy and considered as an indicator of the chemotherapy response.

Study limitations: The relatively small sample size may limit the generalizability of our findings. Additionally, the study population was drawn from a single medical center, which may not reflect the broader demographic and genetic diversity. Furthermore, environmental exposures and lifestyle factors were self-reported, which could introduce recall bias.

Conclusion

This study found that the genotype (AA) of TLR4 (896 A/G) represents 98% of lung cancer cases, genotype (AG) was detected in 2% of lung cancer cases, and the genotype (CC) of TLR4 (1196 C/T) represented 96% of lung cancer cases, while the (CT) allele was detected in 4% of lung cancer cases and 0% of controls. In COPD, the genotype (AA) of TLR4 (896 A/G) represented 100%, with no (AG) allele. The genotype (CC) of TLR4 (1196 C/T) represented 100%, with no (CT) allele. TNF-α was highest in LC patients without chemotherapy and lowest among LC with chemotherapy, indicating a significant negative correlation between chemotherapy and TNF-α.

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